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THERMO-TARGETED DRUG DELIVERY OF GELDANAMYCIN TO HYPERTHERMIC TUMOR MARGINS WITH DIBLOCK ELASTIN- BASED BIOPOLYMERS

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Abstract

The tumor margins are the barrier to hepatocellular carcinoma (HCC) eradication for tumors > 3 cm. Indeed, inadequately treated tumor margins commonly result in local and regional HCC recurrence with increased size and mass. Tumor recurrence is a common problem with chemotherapy, radiotherapy, thermal ablation, and/or surgical resection, by the inability to properly treat the tumor core and the tumor margins. Here we present novel thermosensitive biopolymer-drug conjugates for thermo-targeted chemotherapy at hyperthermic isotherms produced by focal, locoregional thermal ablation. The chemotherapeutic target is heat shock protein 90 (HSP90), a key molecular chaperone of several, and potent pro-oncogenic pathways including Akt, Raf-1, and mutated p53 that is upregulated in HCC. To inhibit HSP90, we have chosen geldanamycin (GA), a potent HSP90 inhibitor. GA has gained significant attention for its low IC₅₀ ~ 1nM and inhibition of Akt and Raf-1, amongst other critical pro-oncogenic pathways. Despite such evidence, clinical trials of GA have not shown promise due to off-target toxicity and poor formulation design. Here, we propose using diblock elastin-based biopolymers as a Ringsdorf macromolecular GA solubilizer - a new generation containing functional poly(Asp)/(Glu) blocks for facile drug conjugation and an ELP block for thermo-targeting of hyperthermic ablative margins. GA release is controlled by pH-sensitive, covalent hydrazone bonds with the biopolymer backbone to avoid systemic toxicity and off-target effects. The resultant biopolymer-conjugates form stable nanoconstructs and display tunable, acute phase transitions at high temperatures. Drug release kinetics are favorable with or without the presence of serum. Thermo-targeted chemotherapy and synchronous thermal ablation provide a unique opportunity for simultaneous destruction of the HCC ablative margins and tumor core for focal, locoregional control of HCC.

Keywords

Elastin-like polypeptide; Heat shock protein 90; Geldanamycin; Hyperthermia; Hepatocellular carcinoma; Tumor (ablative) margin

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Supporting information

¹H-NMR of GA-CHO in *d*₆-DMSO

1. Introduction

Traditional chemotherapy exerts limited efficacy due in large part to poor drug solubility, formulation, off-target toxicity, and plasma instability. Polymer-drug conjugates have been proposed to improve the drug water solubility and supply multiple conjugation sites to maximize the drug loading onto a single macromolecular backbone. In addition, the large macromolecular weight constitutes a Ringsdorf model which provides additional sites for active targeting modalities or therapeutic enhancements (viz. diagnostic agents) [1]. Polymer-drug conjugates also display prolonged plasma half life and may be passively targeted to tumor beds via the enhanced permeability and retention (EPR) effect alone [2]. However, there are still a number of limitations. Synthetic polymer delivery vehicles are often made in toxic organic solvents with long and complex post-reaction purifications. Also, the reaction extent is hard to control between batch to batch, which results in variable and high polydispersity. These limitations are a primary impetus for this study.

Recombinant elastin-like polypeptide (ELP)-based biopolymers, a linear and random coiled polymer, has shown great potential in replicating the Ringsdorf model as previously described, especially for anti-cancer treatment [3-5]. To date multiple ELP-based structures have been developed [6-8]; however, this study presents a unique approach with ELP-based delivery vehicles. ELP consists of pentapeptide repeats with the monomer unit $(GXGVP)_n$ where X (guest residue) can be any amino acid except proline [9]. Without using deleterious solvents, ELP-based biopolymers are designed by protein engineering and expressed in bacteria resulting in high yields, high purity, and low cost for industrial scale-up. In addition, ELP enjoys a modular structural design [10], easy and monodisperse synthesis, and excellent biotolerance [11], which when combined provide for rapid pre-clinical development. Most importantly, for active drug targeting, ELP boasts a unique inverse phase transition behavior upon heating; it remains water-soluble under its thermal transition temperature (T_t) ($T < T_t$), which is fully tunable by biopolymer molecular weight, ELP concentration, and guest residue composition, and collapses at $T > T_t$ forming aggregates. This temperature phase transition behavior is fully reversible and exploited as a non-chromatographical method to purify not only multi-block, ELP-based biopolymers but also fusion products with both high yields and purity [12, 13]. This unique property has been explored to achieve prolonged drug release, enhanced tumor accumulation [14, 15], and delayed tumor progression in tumor models [16]. The rapid thermal transition kinetics of ELP and ELP-based biopolymers promote an exciting course for adjuvant chemo- and/or radiotherapy in conjunction with established clinical thermal ablation or hyperthermic procedures to target the tumor margins, which are the barrier to cancer eradication for tumors > 3 cm [17].

With cancer there is no lack of potential therapeutic targets; however, to promote additive and possibly synergistic effects with a thermal-based delivery system, heat shock proteins (HSPs) have emerged as prime cancer targets. HSPs are shown to be upregulated in heat stressed conditions, such as hyperthermia [18, 19], and overexpressed in many cancer tissues including hepatocellular carcinoma (HCC) [20-24]. HSP90 is a vital molecular chaperone for the correct folding of many oncogenic proteins such as Akt, HIF-1, mTOR, Erb2, and MMP2 [25-28] and highly expressed at the tumor margins [29]. Therefore, the inhibition of HSP90, especially with concurrent application of thermal therapy, holds great potential for multi-targeting various cancer signaling pathways without the complexity of drug-drug interactions, off-target effects, systemic toxicity, or multi-drug resistance [27, 30]. The most potent inhibitor of HSP90 developed so far is geldanamycin (GA) [31]. However, although successful in pre-clinical trials, GA clinical trials have stopped due to extremely poor water solubility and off-target toxicity [32]. A GA derivative, 17-AAG (17-allylamino-17-

demethoxygeldanamycin) has been entered into clinical trials, but the formulation requires toxic Cremophor EL (CrEL) and DMSO to solubilize the drug [33]. More soluble derivatives such as 17-DMAG (17-demethoxy-17-*N,N*-dimethylaminoethylaminogeldanamycin) still suffers from non-tumor specific targeting which requires a delivery strategy to improve its pharmacokinetic profile [34, 35]. Rather than administration of free GA or GA-derivatives, we propose a thermo-targeted biopolymer-GA delivery system to increase drug solubility, avoid off-target toxicities, and specifically target hyperthermic ablative tumor margins with application of thermal ablation lethal to the tumor core. Thermal ablation is the first line nonsurgical treatment option for most HCC patients who are not eligible for surgical resection due to impaired hepatic function [36, 37]. We previously developed a K8-ELP(1-60) system successfully conjugated with GA with significant *in vitro* tumor cell cytotoxicity [38]. ELP nomenclature is presented as “1” representing the guest residue substitution (ELP1 – V5:A2:G3) and “60” the number of pentapeptide repeats. However, the relatively small number of lysine residues limited drug loading capacities. Lysines also provided a four methylene spacer between the active functional group and biopolymer backbone, which increased the length between drugs and ELP backbones resulting in delayed drug release kinetics [5]. Furthermore, the low molecular weight of the total construct (25.4 kDa) might not provide sufficient stealth properties, thus limiting evasion of the reticuloendothelial system (RES) and the degree of EPR targeting. Here, we present the next generation of ELP-based biopolymers comprised of 15 aspartic (D) or 15 glutamic acids (E) and ELP(1-60/90/180) block. E15-ELP(1-90) was chosen as the initial candidate for GA conjugation due to its higher $T_t \sim 40^\circ\text{C}$, (pertinent to microwave antenna *in silico* and *ex vivo* $T > 40^\circ\text{C}$ data at peripheral isotherms > 3 cm away from the microwave antenna (data not shown); a sublethal temperature but sufficiently adequate for thermo-sensitive chemotherapy via E15-GA-ELP(1-90) biopolymer-conjugates). A covalent, pH-sensitive hydrazone bond was designed to release GA specifically within the acidic tumor microenvironment or within the acidic endosomes or late lysosomes of tumor cells [39], which is yet another active targeting modality against the tumor margins. We were able to obtain covalently conjugated GA in nearly 50-67% capacity while retaining thermal-responsive properties to target hyperthermic tumor margins with clinically established MTA.

2. Materials and Methods

2.1 Reagents

Oligonucleotides were synthesized at the Research Core Facility, University of Utah (UU). DH5 α cells and fetal bovine serum (FBS) were purchased from Invitrogen (Carlsbad, CA); pET25b+ expression vector and *E. coli* BLR (DE3) strains were obtained from Novagen (Madison, WI). T4 DNA ligase, restriction enzymes, calf intestinal alkaline phosphatase (CIP), and pUC19 cloning vector were purchased from New England Biolabs (Beverly, MA). Circle-Grow culture medium was purchased from Q-BIOgene (Carlsbad, CA). Geldanamycin (GA) was obtained from LC Laboratories (Woburn, MA) and structure confirmed upon receipt by $^1\text{H-NMR}$. Carbenecillin sodium salt was purchased from ISC Bioexpress (Kaysville, UT). Aminoacetaldehyde diethyl acetal (AAADA), chloroform, carbohydrazide, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC)-HCl, sulfo-*N*-hydroxysuccinimide (NHS), and dimethyl sulfoxide (DMSO) were from Sigma-Aldrich (Milwaukee, WI) and used as received.

2.2 Construction of Diblock Elastin-like Biopolymers: D15- or E15-ELP(1-60/90/180)

First, oligos encoding 15 aspartic (D15) or 15 glutamic (E15) acids were designed with ApE software, optimized for bacterial expression, and synthesized by the UU Core Facility:

- D15 forward: 5'-gtggacgatgatgacgatgatgatgacgatgatgatgacgatgatggtggc-3'

- D15 reverse: 5'-accatcatcatcgtcatcatcatcgtcatcatcatcgtccacgcc-3'
- E15 forward: 5'-gtggaggaagaagaggaagaagaagaggaagaagaagaggaagaaggtggc-3'
- E15 reverse: 5'-accttcttctcttcttcttcttcttcttcttcttcttcttcttcttcttccacgcc-3'

The oligos were annealed to form a double-stranded DNA cassette with *Pf*MI compatible ends at the 5'. Second, ELP(1-60/90/180) were polymerized from ELP(1-10), where “1” stands for a “X” guest residue substitution of V5:A2:G3, “10” represents 10 amino acids in total, through recursive directional ligation (RDL) [40]. Both agarose gel electrophoresis and DNA sequencing verified correct insertions. The validated ELP(1-60/90/180) pUC19 plasmids were further digested with *Pf*MI restriction enzymes, dephosphorylated by CIP, and ligated with annealed D15/E15 oligos. The cloning scheme starting from ELP homopolymer is illustrated in Figure 1. Sequence verified plasmids were transformed into DH5 α cells by heat shock. After amplification at 37°C overnight, plasmids were purified using a QIAGEN Miniprep Kit (Valencia, CA), double digested with *Nde* I and *Hin*D III, and ligated to compatible pET25b+ expression vectors to yield D15- or E15-ELP(1-60/90/180) expression vectors. The expression vectors were transformed into *E. coli* BLR (DE3) strain by heat shock. The diblock copolymers were expressed at 37°C for 9 h at 230 rpm, followed by shaking incubation at room temperature for another 15 h. After the removal of cell lysate by centrifugation and sonication, the diblock biopolymers were purified by inverse transition cycling (ITC) [41] with pH adjustment to neutral using 1M HCl before the first hot spin. The purity and molecular weight of the biopolymers were confirmed by tris-glycine SDS-PAGE and mass spectrometry analysis. The biopolymers were lyophilized and stored at -20°C for future use.

2.3 Thermal transition temperature profile of D15/E15-ELP(1-60/90/180) diblock biopolymers

Inverse phase transition is the key parameter to realize thermo-targeted drug delivery. It has been demonstrated that the thermal transition temperature (T_t) of the ELP-homopolymer is affected by ELP length, concentration, and hydrophobicity [40]. This generation of ELP-based diblock biopolymers is entirely unique with the addition of 15 consecutive hydrophilic amino acids; hence, thermal transition profiles demonstrate new features distinct from ELP homopolymers. The T_t as a function of ELP length, concentration, and pH was measured using a CARY 100-Bio UV-Vis spectrophotometer (Palo Alto, CA). The optical density at 650 nm was monitored at 1°C/min temperature increments in the range of 25-75°C. The T_t was determined as the temperature at the maximum derivative of turbidity at the wavelength of 650 nm. This wavelength was chosen to avoid the influence of high absorption from GA.

2.4 Activation of geldanamycin (GA) and D15-ELP(1-60) and E15-ELP(1-90) biopolymers

GA is a potent HSP90 inhibitor extracted from *Streptomyces hygroscopicus* [42]. To retain biofunctionality following bond cleavage from the biopolymers, the 17-methoxy was converted to an aldehyde using amino acetaldehyde diethyl acetal (AAADA) as described elsewhere [38]. The final purple colored product was washed with anhydrous chloroform, which was eliminated by overnight rotary evaporation. Product purity was confirmed by TLC and ¹H-NMR (*d*₆-DMSO) (supporting information). The carboxyl groups of the diblock biopolymers were converted to amines using carbohydrazide. Briefly, freeze-dried copolymers were dissolved in 0.1M sodium phosphate buffer (10-20 mg/mL) supplemented with 32 mg/mL carbohydrazide. EDC (50 mM) and sulfo-NHS (5 mM) were then added and stirred at room temperature (450 rpm) for 4 h. The activated diblock copolymers were purified by size exclusion chromatography (SEC) using a PD-10 column eluted with water. Products were lyophilized and stored at -20°C.

2.5 Hydrazone conjugation of GA to ELP-biopolymers

25 mg of activated diblock biopolymer was mixed with activated GA-aldehyde (molar ratio 1:2) in anhydrous DMSO as shown in Figure 2. The reaction mixture was stirred at 23°C for three days and the product was then dialyzed in water for two days to eliminate residual solvent. Insoluble GA-aldehyde was further removed by both centrifugation and PD-10 SEC. The final conjugated product was lyophilized for future characterization. The GA:biopolymer conjugation ratio was determined by a UV-Vis calibration curve and further confirmed by MS. To reduce the space for nomenclature, we will use GA-D15-*n* or GA-E15-*n* for GA diblock biopolymer conjugates with hydrazone linkages, where “*n*” stands for the total number of total amino acids for ELP(1-*n*) library (60 or 90).

2.6 Physical stability of GA-ELP biopolymer conjugates

2.6.1 Particle size distribution and zeta potential analysis—Diblock-biopolymer and GA conjugates were dissolved in 0.8 mL PBS and purified through a 0.2 µm filter with the final concentration ranging from 10-25 µM determined by a UV-Vis calibration curve. Particle size was measured at 25°C using a BI-200SM, dynamic light scattering (DLS) system (Holtsville, NY). Particle size was also measured in PBS containing 20% FBS to mimic *in vivo* stability. Particle size distribution was further validated by transmission electron microscopy (TEM). Briefly, small drops of purified biopolymers and conjugate solutions (25 µM) were air-dried on carbon-coated copper electron microscopy grids for 10 min. Protein nanoparticles were negatively stained with 2% phosphotungstic acid for 30 s and excess liquid was removed by filters. Grids were examined in a TEM (PHILIP) at 120 kV and resulting images were acquired by a charge-coupled digital camera. Concurrently, the zeta potential of the biopolymer-GA drug conjugates was measured at the same conditions using a ZetaPALS zeta potential analyzer at 25°C (Holtsville, NY). Data for both sets represent three independent trials.

2.6.2 Thermal transition temperature profile of GA biopolymer conjugates

The hydrophobicity of the GA directly affects the macromolecular assembly of the biopolymer-GA conjugates; therefore, the thermal transition behavior at the same ELP length and concentration would be expected to be different. The thermal responses of conjugates were measured both in PBS and 20% FBS as monitored by UV-Vis at wavelength of 650 nm.

2.7 Drug release profile of GA-ELP biopolymer conjugates

The pH release kinetics at both acidic and neutral pH were performed on an Alliance RP-HPLC equipped with an absorbance reader. 25 µM GA-E15-90 samples (concentration based on the ELP) were prepared in 1 mL of PBS at pH 4.5 or pH 7 and were shook at 37°C or 45°C at 180 rpm. 100 µL samples (cooled on ice first) were loaded at 0, 3, 6, 12, 24, 48, and 72 h with a 75 µL injection volume. GA-E15-90 and free GA were separated on a diphenyl column (Waters) using a H₂O/90% DCM gradient with 0.1% v/v TFA. Initially, the binary gradient was 70:30 for 10 min at which point the 90% DCM concentration was raised to 60% over 30 min followed by a static 10 min period. The flow rate was 1.0 mL/min. Percent of GA release as a function of time was based upon a drop in the GA-E15-90 peak area percent, normalized to the 0 h time point [5]. These runs were repeated three times for each GA-E15-90 conjugate.

3. Results and Discussion

An ELP(1-*n*) library starting from the ELP(1-10) monomer was successfully cloned using the RDL approach, as delineated in Figure 3. Insertion of D15 or E15 blocks was confirmed by an additional sequencing step in both the pUC19 vector, and the subsequently subcloned

pET25b+ vector, which was eventually transformed and allowed to express proteins in BLR (DE3) cells. The typical purification steps of E15-ELP(1-90) diblock copolymer are described in Figure 4. A slight modification was made before the first hot spin step due to the insertion of ample carboxyl groups, whose protonation creates a stiff hydrogen bonding block leading to higher transition temperatures. Typically two rounds of ITC were sufficient to obtain products with high purity, as suggested by the SDS-PAGE. Due to the random coil, linear structure of the polypeptide, ELP-based biopolymers showed higher molecular weights than expected (>40 kDa estimated by the gel), therefore, the precise molecular weight was further validated by electro-spray ionization mass spectrometry (ESI-MS) (Figure 5). Using the simple and efficient purification methods, a large library of ELP diblock copolymers were constructed and summarized in Table 1.

The ELP diblock biopolymers displayed interesting thermal transition behaviors. Figure 6A demonstrated that T_t was not a function of concentration for D15-ELP(1-60), or any other copolymers we have made, which is not the case for ELP alone.[43] We propose that the addition of 15 more hydrophilic amino acids promotes the self-assembly of the copolymer to a micelle-like structure, where the intramolecular hydrophobic interactions are greatly enhanced. This hypothesis remains to be validated. The proposal is further supported by the DLS data and TEM image in Figure 7A where spherical structures with size ca. 50 – 100 nm were observed. On the other hand, T_t remains to be a function of molecular weight suggested by Figure 6B – T_t dropped as the length of ELP-block increased. T_t shows strong dependence on pH where T_t increased with the decrease of pH values (Figure 6C) except for the point approximate to the pKa of glutamic/aspartic acid where precipitation might occur to reduce the apparent concentration. We believe that the ample carboxyl groups exposed to the water interface can form stiff intermolecular hydrogen bonds that require more heat to destabilize the supramolecular self-assembled structures.

Drug conjugation ratios of GA-conjugates were confirmed by UV spectrometry and MS. As shown in Figure 8, GA-E15-90 showed a significant increase in UV absorbance at 337 nm, which is the distinctive absorption peak for GA-CHO. The drug loading was calculated using a calibration curve of both GA-CHO and ELP diblock biopolymers. We show 46-67% of the available sites successfully conjugated onto the D/E side chains. Drug content was further confirmed by MALDI-TOF MS where a significant shift of the molecular weight was obtained (Figure 9). Since the GA-CHO is very hydrophobic, the high drug conjugation ratio could facilitate a core-shell self-assembly of the conjugate, where the drug is shielded in a hydrophobic core away from the aqueous milieu, while the ELP homopolymer blocks are exposed to the water interface. We have observed that higher drug conjugation ratios result in a narrower size distribution as seen in Figure 10A, where 10 out of 15 available hydrazides on the activated D15-ELP(1-60) were occupied by GA-CHO; whereas the E15-ELP(1-90) counterpart, which has 20% less degree of conjugation demonstrated a higher polydispersity as indicated in Figure 10B. Both constructs displayed a slightly positive charge (+2.6-3.3mV) suggesting high drug conjugation and stable nanostructures have been formed. The ample carboxyl group exposed to the water resulted in a negative surface charge (-20 ± 2.9 mV) of ELP diblock biopolymer. After activation with the carbohydrazide, the zeta potential was converted to the positive by the attached amine groups ($+16 \pm 3.3$ mV) not conjugated to GA. The charges were further reduced through the conjugation of multiple GAs which form neutral hydrazone bonds. It has been shown that nanoparticles with slight surface charge achieve higher accumulation in tumors [44] as well as less systemic aggregation [45, 46]. We are optimizing the preparation methods to engineer even smaller nanoparticles.

The transition profiles of the GA conjugates are summarized in Figure 11. Similar independence of concentration on T_t was observed for all three conjugates; however, a

moderate concentration of conjugate ($> 25 \mu\text{M}$) may be needed to ensure fast transition kinetics. As the molecular weight of the ELP block decreases, a higher T_t was obtained. The influence of serum on the transition behavior is reported in Figure 12. The transition kinetics were slightly lower due to the mixed population proteins; however, it is to our advantage that the T_t increases after the addition of serum which reaches hyperthermic temperature ($>41^\circ\text{C}$). In order to test the reproducibility of the thermal transition, we conducted two consecutive thermal runs under the same experimental conditions. The second thermal transition is affected by the serum due to some aggregation of ELP with serum proteins as suggested by the downscan of the first run. However, the thermal kinetics are still retained despite the presence of serum. Rapid aggregation of ELP can be achieved simply by increasing the concentration of the conjugates. Further studies are ongoing evaluating the length of polyD/E and ELP composition to tailor the transition temperature by promoting intramolecular hydrophobic interactions.

The release of GA-CHO as a function of time from copolymer conjugates was assessed in PBS buffer at pH 4.5 and pH 7.4. GA-E15-90 (R_t 26 min), ELP copolymer (R_t 22 min), and free GA-CHO (R_t 18 min) can be separated using the binary gradient in a 52 min run. By calculating the percentage drop in the GA-E15-90 peak area, over 60% GA is considered to be released from the ELP backbone, while only about 20% of GA is released at neutral pH over the three-day time frame. (Figure 13) In order to understand the release kinetics at hyperthermic conditions, we also conducted release study at 45°C at both pH for three days. The release is almost complete at pH 4.5 with much faster kinetics than the release at the 37°C . Interestingly, the release at neutral pH is also greatly increased by the high temperature. It has been found that hydrolysis kinetics of hydrazone is much higher at high temperatures [47-49]. Kovariková *et al* have also demonstrated that the break-down rate of hydrazone bonds increases threefold at 40°C [50]. The significant release at even neutral pH will not cause unwanted side effects since the drug is still released at ablated/tumor margins where the particles have hydrophobically aggregated. We are optimizing the ELP structures to achieve even faster drug release at 45°C under the presence of serum. The fast kinetics at high temperature will ensure an acute killing of cancer cells before the expression of heat shock proteins which might account for focal, locoregional tumor recurrence.

4. Conclusion

We have successfully constructed a new breed of ELP-based diblock copolymers featuring poly glutamic/aspartic acids for facile drug conjugation and thermo-targeted chemotherapy of hyperthermic tumor margins. D15/E15-ELP(1-60/90) polymers were conjugated with GA-CHO and high drug loadings were achieved with retained thermo-responsiveness and particle stability with a unimodal size distribution. The addition of serum proteins does not significantly perturb the thermal transition behavior. Thus, the ELP-based polymer-GA conjugates demonstrate great potential for thermo-targeted chemotherapy of the tumor margins and inhibition of HSP90 at these sites with further synchronous use of MTA. Active targeting will result by thermal precipitation of the biopolymer-GA conjugates at hyperthermic isotherms $T > 40^\circ\text{C}$ (verified by *in silico* and *ex vivo* correlation) and pH-sensitive drug release within the acidic tumor microenvironment; passive targeting may occur by the putative EPR effect. Further studies will delineate drug release kinetics *in vitro*, characterization of the self-assembled particles, and establishment of an *in vivo* tumor model suitable for synchronous thermo-sensitive drug delivery and MTA. The versatility of our cloning schemes ensures fast optimization of polymer composition. In addition, the controlled synthesis and purification of the biopolymer, armed by high drug loading potentials, tunable thermo-responsiveness, and particle stability further presents a novel platform promising for advanced anti-cancer treatment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

A typical round of RDL for homopolymer cloning. “1” stands for a “X” guest residue substitution of V5:A2:G3, “n” stands for total number of amino acids comprising ELP, “m” indicates total aspartic(D)/ glutamic(E) acid repeats. Further insertion of D/E15 components was realized using *PflMI* restriction enzyme. The final construct was transferred to a pET25b+ plasmid for expression.

Figure 2. Chemical schemes of geldanamycin (GA) activation (A) and conjugation with E15-ELP(1-90) through pH-sensitive hydrazone linkages (B). 1, Geldanamycin (GA); 2, aminoacetaldehyde diethyl acetal (AAADA); 3, acetal modified GA by-products; 4, aldehyde-modified GA (GA-CHO); and 5, GA-E15-90.

Figure 3.

ELP(1-*n*) homopolymer libraries. The expected band lengths are denoted at the right panel of the gel picture. Gel composition and running conditions: 1.8% agarose, 70V, 3h.

Figure 4.

A typical 4-20% Tris-glycine SDS-PAGE of E15-ELP(1-90) run at 90 V for 150 min stained with Invitrogen safeblue®. Samples from lanes 2-7 were taken after each purification step designated in the box. ELP(1-90) was also loaded as a control for purity verification. A MW shift indicates the insertion of the new poly(E) block.

Figure 5. Electrospray ionization mass spectrometry (ESI-MS) of E15-ELP(1-90). The molecular weight is in accordance with the expected value (38328.6 Da).

Figure 6. Temperature transition behaviors of ELP-diblock copolymers. (A) T_t under different concentrations of D15-ELP(1-60); (B) T_t as function of molecular weight; (C) pH influence on T_t . Both D15-ELP(1-180) and E15-ELP(1-90) display the same pattern of increased T_t at lower pH.

Figure 7. Transmission electron microscopy (TEM) images of E15-ELP(1-90) copolymer (A) and GA-D15-60 (B). The image shows a particle size ca. 50-100 nm for the native copolymer and 100-150 nm for the conjugate, particle sizes lower than that obtained from dynamic light scattering (DLS) presumably due to dehydration.

Figure 8. UV-Vis profile of GA-E15-90 conjugate. The distinct peak at wavelength of 337 nm demonstrates a high conjugation ratio of GA-CHO to the activated ELP bio-copolymer.

Figure 9. MALDI-TOF mass spectrometry for GA-E15-90 conjugate. The molecular weight shift indicates approximately seven GA molecules per ELP-biopolymer.

Figure 10.

(A) Size distributions of GA-D15-60 conjugate; (B) E15-ELP(1-90) copolymer and GA-E15-90 conjugate. All samples were measured three times in PBS (25 μ M), data represented with standard error of the mean. The GA-E15-90 conjugate (drug ratio 46.7%) shows a bi-modal distribution with a small portion of free copolymer. The GA-D15-60 demonstrates a narrower size distribution due to higher drug conjugation ratios (66.7%).

Figure 11.

Thermal transition behavior of GA-polymer conjugates. For GA-E15-90, 50 μ M is sufficient for rapid thermal kinetics. The effect of concentration on T_t is again not pronounced, which is similar to the effect on the ELP copolymer. T_t can be increased by shortening the ELP block length.

Figure 12.

Thermal transition behavior of GA-E15-90 in 20% serum (25 μ M). Comparable kinetics have been achieved with slightly increased T_t which is beneficial for hyperthermic, tumor margin aggregation at $T > 40^\circ\text{C}$. The thermal profile was repeated once under the same conditions. (2nd runs) Similar thermokinetics and T_t have been achieved.

Figure 13. GA release from GA-E15-90 (25 μ M in PBS) as function of time at pH 4.5 and pH 7.4. Release profile at both 37 °C and 45°C were recorded. Data reported as mean $\pm$ S.D., n =3.

Table 1

Summary of ELP-based diblock copolymers expressed. Transition temperatures were calculated at normalized concentration of 25 μ M in PBS.

Biocopolymer amino acid sequence	Monomers	Mw (KDa)	Yields (mg/L culture)	Tt ($^{\circ}$ C) (25 μ M)
MWGHGV-(E) _m -H-(GXGVP) _n -GGLK	m (15) n (90,180)			
(E) _m -ELP(1- <i>n</i>)	E15-(1-90)	38.3	15	35.5
	E15-(1-180)	73.5	20	33.0
MWGHGV-(E) _m -H-(GXGVP) _n -GGLK	m (15); n(60, 90,180)			
(D) _m -ELP(1- <i>n</i>)	D15-(1-60)	26.3	15	39.1
	D15-(1-90)	38.1	20	37.0
	D15-(1-180)	73.2	40	34.0